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The Role of Traf2 in Zebrafish

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THE ROLE OF TRAF2 IN THE ZEBRAFISH

by

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Abstract:

Tumor necrosis factor (TNF) receptor associated factor 2 (Traf2) is suspected to be involved in the anti-apoptotic pathways of the innate immune system in the zebrafish. The expression of three different forms of Traf2, 2a1, 2a2, and 2b, was knocked down in pairs using antisense morpholino oligonucleotides in zebrafish embryos. A rescue was attempted by knocking down a potential receptor for the Traf2 to pathway. After infection with snakehead rhabdovirus (SHRV), mortality was quantified and the apoptotic effects of each of the Traf2 genes was assessed. Cell death was quantified using the TUNEL technique. The findings indicated that at least two zebrafish Traf2 proteins were necessary for function. Increased mortality due to infection was not caused by an increased amount of virus in the Traf2 morphants or increased amount of apoptosis.

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Introduction

We are inundated every day by viruses. Ranging from the common cold to sexually transmitted diseases, everyone may be impacted by a viral infection at some point in their lives. The general viral infection process occurs when the virus is endocytosed by the cell. Once the virus enters, the nucleic acid of the virus is released into the cell cytoplasm to utilize host cell proteins for replication. As a protective mechanism, host cell proteins such as Toll-like receptors, recognize the DNA or RNA of the virus as foreign to the host. This initiates a protein signaling cascade that ultimately results in the translocation of transcription factors into the nucleus to transcribe pro-inflammatory cytokines. Production of pro-inflammatory cytokines is the cell's way of defending against the viral infection.

The immune system is the body's defense against pathogens of all kinds, like viruses. There are two major branches: the innate immune system and the adaptive immune system. The adaptive immune system takes up to ten days to develop an appropriate response, but is then more specific to the pathogen and has a memory of the pathogen. It is composed of specialized white blood cells known as B and T-cells. The innate immune system protects the body during those first seven to ten days. Without a vigorous innate immune response, the adaptive immune response will not be as strong. The innate immune system provides a general response and is composed of the skin, mucous membranes, and various phagocytic cells. Generalized cell receptors, known as Toll-like receptors (TLRs), are responsible for recognizing the invading pathogens and initiating protein cascades to help cells deal with the infection. TLRs 3, 7, 8, and 9 are involved in viral recognition.

In mammalian systems, TLR3 uses a TICAM1 dependent pathway to initiate the protein cascades, while TLRs 7-9 use a MyD88 dependent pathway. Zebrafish do not possess a protein homolog to TICAM1, and thus can only use MyD88-dependent signaling through the TLRs. To find potential signaling partners for MyD88 in zebrafish, a pulldown assay was conducted using zebrafish MyD88 as bait (unpublished data from Charette *et al*). A potential signaling partner for MyD88 was found to be Traf2 in this pulldown.

One of the adaptor proteins in the mammalian TLR3-TICAM1 dependent pathway in mammalian systems is Traf2, a member of the tumor necrosis factor receptor associated factor protein family. In mammalian systems, Traf2 does not associate with MyD88, but based on the previous findings in the Kim lab, we hypothesized that Traf2 may be associating with MyD88 in a unique interaction not seen in mammalian systems. If Traf2 is interacting with MyD88 in zebrafish signaling pathways this would be a unique and novel interaction not seen in any mammalian systems. For my senior thesis project, I examined the potential role of Traf2 in the zebrafish immune system.

Literature Review

Zebrafish as a model organism

During the 1960s, Dr. George Streisinger developed a new model organism for studying human diseases: the zebrafish (35). Over the next half century, this new model organism exploded with possibilities for research. A quick summary of some of these major landmarks is summarized in Figure 1 (adapted from 18). These early years were

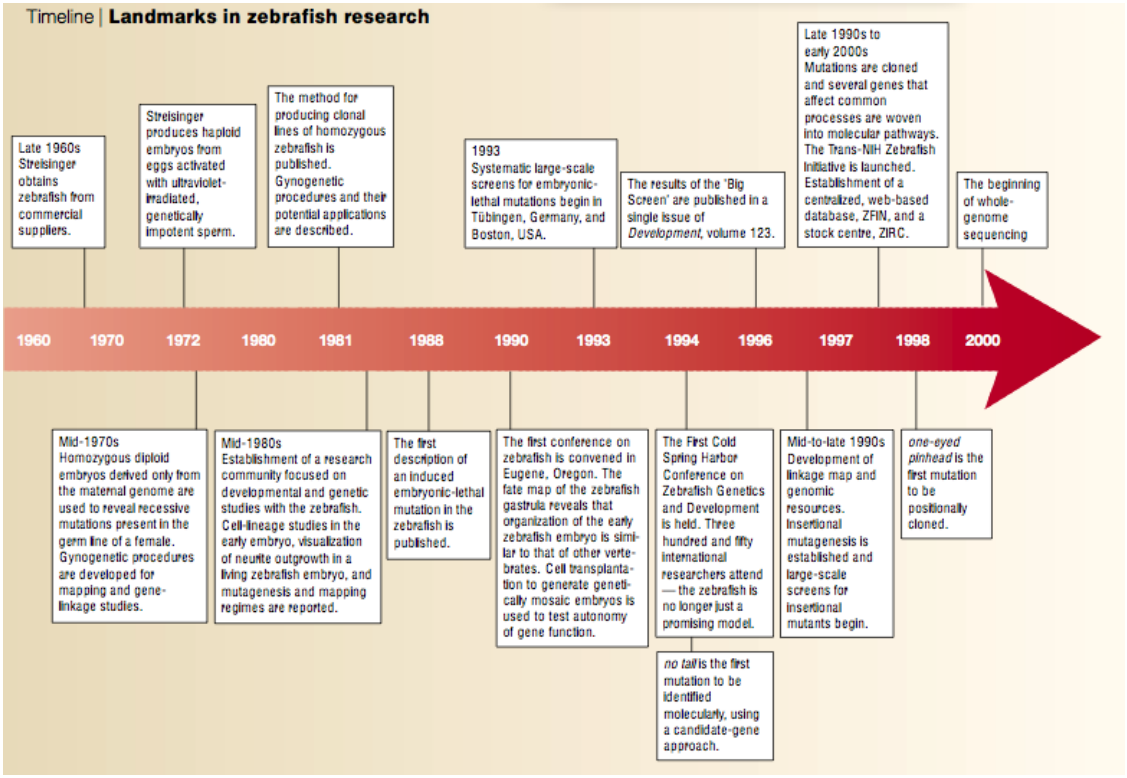

Figure 1: ***Major developments in zebrafish research*** **(18).**

focused on genes and genetics, especially since transcription and translation mechanisms were just being deduced. Streisinger was at the forefront of the genetics work with phages, an interest that naturally led him to mutational analysis in vertebrates (18). Additionally, Steisinger was known as a fish hobbyist. Trials with a variety of tropical fishes left zebrafish as the prime candidate over other fish such as the medaka – with previously developed genetic experimentation – due to their prodigious breeding, external fertilization, and clear embryos (18). His work during the 70s and 80s produced haploid embryos and homozygous diploid clones, throwing zebrafish into the limelight (35).

The zebrafish (*Danio rerio*) is a common aquarium fish and excellent model organism for molecular research. The externally fertilized embryos develop in optically clear shells. The large clutch size and short generation time allow larger studies than more conventional model organisms such as mice (8, 14). Embryogenesis is completed by 50 hours post fertilization (hpf). Four days post fertilization (dpf) the adaptive immune system begins developing and is fully functional four to six weeks after fertilization (8, 46). The zebrafish genome is completely sequenced and many molecular tools such as morpholinos are readily available to manipulate the genome. Other molecular tools include zinc finger nucleases, highly homozygous strains of zebrafish, and transgenic fish (14, 46).

Zebrafish are excellent models for studying the immune system, particularly innate immunity (35). A variety of pathogens and even some parasitic organisms such as dinoflagellates have been used to replicate infections in human systems and set zebrafish as a model for infectious disease (Table 1) (35, 55).

Table 1: ***Pathogens infecting zebrafish to model infectious diseases*** **(35).**

Pathogen	Infection route	Model	Pathophysiology	Refs
Gram negative				
Aeromonas salmonicida	(A) Application to scraped dermis	Demonstrated a mammalian-like acute-phase response	Septicemia and death	[65]
Edwardsiella tarda	(E) Static immersion; (A) immersion following dermal scraping or i.p. injection	Demonstrated a mammalian-like acute phase response	Septicemia and death	[66]
Salmonella typhimurium	(E) i.v. injection into axial vein	Evaluated pathophysiology using fluorescent bacteria	Bacteria replicate intravascularly and within macrophages leading to overwhelming infection	[67]
Salmonella arizonae	(E) Injection	Evaluated pathophysiology	Replicate in macrophages leading to lysis; death of embryo	[68]
Vibrio anguillarum	(E) Static immersion	Evaluated pathophysiology	Infection of GI tract and skin	[69]
Gram Positive				
Staphylococcus aureus	(A) i.p. injection	Demonstrated a mammalian-like acute phase response	Septicemia and death	[65]
Streptococcus iniae	(A) i.m. injection in dorsal muscle; i.p injection	Bacterial mutagenesis screen	Localized skin cellulitis, systemic infection with multiple organs, sepsis, meningoencephalitis, death.	[70,73]
Streptococcus pyogenes	(A) i.m. injection in dorsal muscle; i.p injection	Assessed pathogenesis of bacterial mutants	Locally spreading disease similar to necrotizing fasciitis	[70,73,75]
Listeria monocytogenes	(A) i.p. injection	Evaluated pathophysiology	Unable to multiply within ZF	[76]
Bacillus subtilus	(E) i.v., intracardiac or intra-ventricular injection	Evaluated role of macrophages	Macrophages rapidly phagocytose bacteria	[43]
Mycobacterium marinum	(E) Static immersion or injection; (A) i.v. or i.p. injection	Evaluated pathophysiology using fluorescent bacteria	Granulomatous disease similar to tuberculosis	[68,77,78]
Viruses				
Infectious hematopoietic necrosis virus and infectious pancreatic necrosis virus	(A) i.p. injection	Assessed pathogenic response on blood formation	Transient toxic effects on kidney marrow with spontaneous recovery without sequelae	[79]
Spring viremia of carp virus	(A) Immersion	Determined increased pathogenic response in colder temperatures	Multifocal necrosis with high mortality	[80]
Snakehead rhabdovirus	(E) and (L) immersion; (A) i.p. injection	Demonstrated up-regulation of IFN and Mx	Necrosis of pharyngeal epithelium and liver with high mortality	[81]
Viral hemorrhagic septicemia virus	(A) i.p injection of immersion	Demonstrated efficacy of vaccination with attenuated virus	Hemorrhage in muscle and liver with high mortality	[82]
Nervous necrosis virus	(L) Injection; (A) i.p. injection	Demonstrated importance of IFN response for mortality	Brain lesions with high mortality in larvae but more chronic infection in adults	[83]

Abbreviations: (E) = embryo; (L) larvae; (A) = adult; ZF = zebrafish.

Snakehead Rhabdovirus

Snakehead rhabdovirus (SHRV) is in the virus family *Rhabdoviridae*. It is enveloped and contains a negative-sense RNA genome. Originally cultured from infected snakehead fish, SHRV is effective in inducing viral infections and in zebrafish induces a cytokine response (35, 40). SHRV replicates at temperatures between 24 and 30 ^{o}C, an ideal temperature since zebrafish are maintained at 28 ^{o}C. Phelan *et al* demonstrated an effective infection technique for embryos and adult fish alike using immersion in egg water and virus (40). Immersing embryos from 24 hours post fertilization (hpf) to 3 days post fertilization (dpf) in 10^6 $TCID_{50}$ SHRV/mL for 5 hours and then washing the fish induced 55% and 47% mortality, respectively (8). After initial immersion, the virus invaded and spread throughout the fish and induced interferon production, a characteristic of viral infection (8).

Innate Immunity

The immune system is compromised of two major branches: innate immunity and adaptive immunity. The innate immune system provides a first line of defense against pathogens with a immediate, non-specific response (27). In contrast, adaptive immunity has a specific response and possesses memory. The memory of the adaptive immune system provides a stronger response the second time a host is exposed to the pathogen (27).

The two branches are linked in many ways and complement each other's function (27). Innate immunity, the focus of this study, recognizes broad classes of pathogens with receptors that interact with proteins on the pathogen's surface. The toll-like receptors (TLRs) are a family of transmembrane proteins that function within the innate immune

system as pattern recognition receptors (PRRs) (2). The PRRs are responsible for recognizing the pathogen associated molecular patterns (PAMPs), which are ligand motifs present on the outside of a pathogen surface. PAMPs, such as LPS and peptidoglycan, are recognized in general by class by the PRR. The binding of PRR-PAMP initiates the TLR signal transduction pathways involved in the elimination of the pathogens, as appropriate for the general class of antigen. Activation of TLRs leads to the activation of NF-κB and induction of cytokines and interferons (1, 45). The TLR pathways that lead to cytokine induction have been characterized in mammals, including humans (42); however, in lower vertebrates, such as zebrafish, the TLR signaling pathways have not yet been defined (42, 45).

Toll-like receptors

Toll-like receptors (TLRs) were originally discovered in *Drosophila* as a

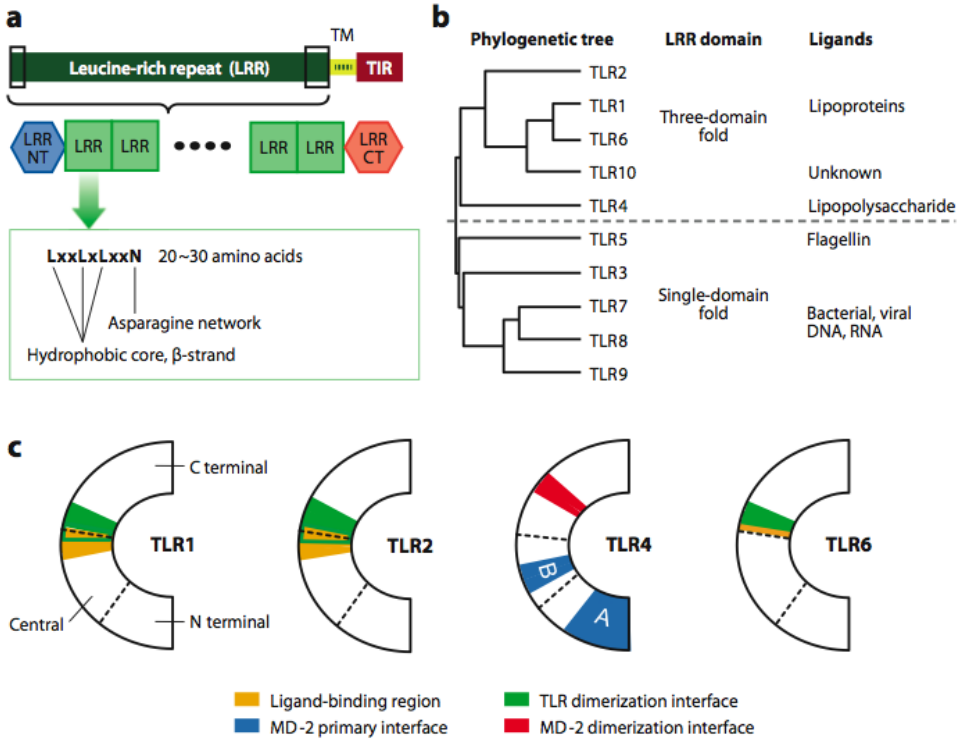

Figure 2: *General structure of TLRs*. A. Domains of general TLR. B. Phylogenetic tree of TLRs and the PAMPs recognized. C. Differences between specific TLRs (24).

mechanism to resist microbial infections and as an important protein in development (2, 27). An extracellular leucine-rich repeat region (LRR) and an intracellular Toll/interleukin-1R homology (TIR) domain create the generalized TLR structure (Figures 2, 3) (24, 27). The LRR is made up of the repeating amino acid motif LxxLxLxxN and are characterized by a horseshoe solenoid tertiary structure (Figure 3) and β sheets within the concave section of the horseshoe (9, 24).

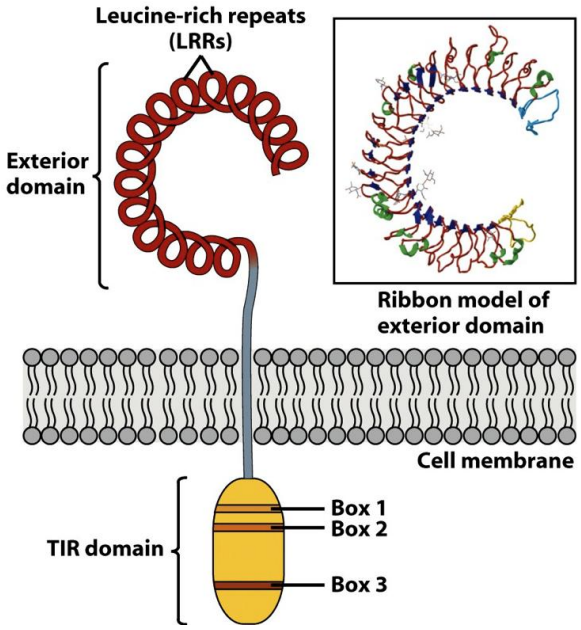

Figure 3: ***Structure of TLRs*** **(27).**

TLRs can homo- and heterodimerize with other TLR molecules (27). Intracellular signaling takes place through adapter molecules, such as MyD88 and TICAM, that bind the TLR TIR domain via their own TIR domain (24).

TLRs recognize a variety of PAMPs, from lipoproteins to viral RNA (2). TLR1, -2, -4, -5, and -6 are on the cell surface and recognize bacterial parasites, gram-positive and gram-negative bacteria, flagellated bacteria, and fungi. Embedded in intracellular vesicles, TLR3, -7, -8, and -9 recognize viral dsRNA and ssRNA in addition to certain bacterial DNA elements (Figure 4) (27).

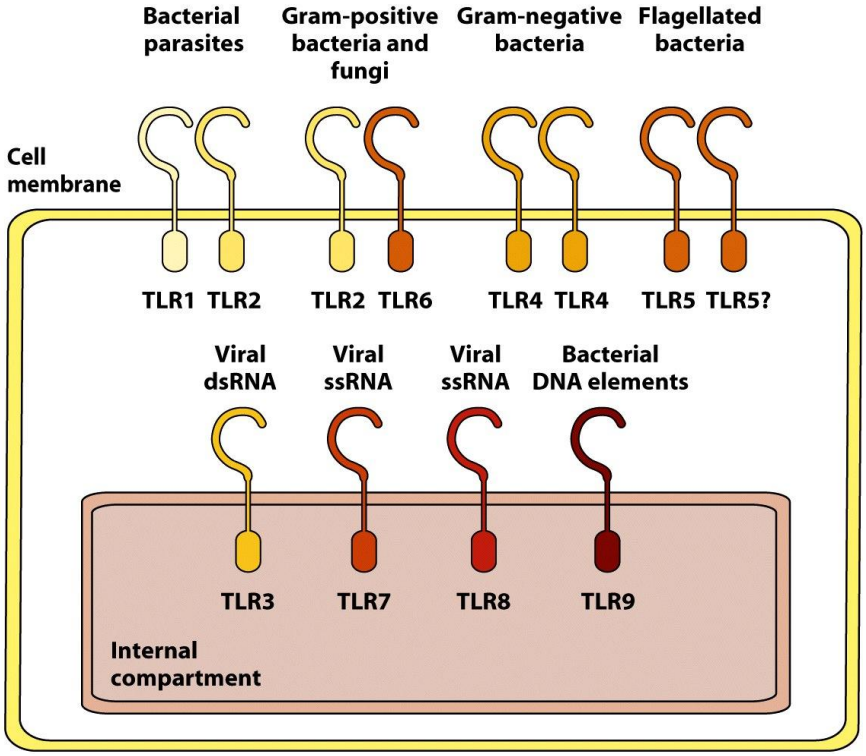

Figure 4: ***Distribution of TLRs throughout the cell and major PAMP recognized by each TLR*** **(27).**

TLRs are involved in a wide variety of cell signaling. An overview of some of the pathways they participate in is shown in Figure 10 (44). Though all the pathways are initiated by different signals, such as flagellin or dsRNA, ultimately, the end is the same: apoptosis or inflammatory and immune gene activation.

Apoptosis versus Necrosis

Cellular death is a highly controlled and moderated process within cells. The condensing and breakage of DNA and the creation of apoptotic bodies characterize apoptosis, or programmed cell death, whereas

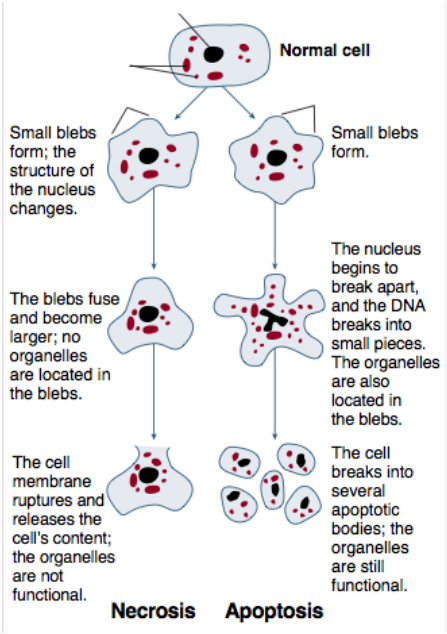

Figure 5: ***The differences between necrosis and apoptosis*** **(36).**

necrosis is characterized by membrane blebbing and the dumping of cellular contents (17, 36). The terms associated with and utilized in cellular death are listed in Table 3 and summarized with their major characteristics (17). Though not listed in the table, necrosis is often associated with tissue damage. Apoptosis and necrosis can be difficult to distinguish, especially since the same stimuli at different doses can induce both types of cell death (15). Table 2 summarizes some of the characteristics between apoptosis and necrosis (15). The major difference is the rupturing of the cell membrane during necrosis that eventually leads the loss of the cytoplasm. Inflammation is another key difference present in necrosis but not in apoptosis (15).

Apoptosis	Necrosis
Single cells or small clusters of cells	Often contiguous cells
Cell shrinkage and convolution	Cell swelling
Pyknosis and karyorrhexis	Karyolysis, pyknosis, and karyorrhexis
Intact cell membrane	Disrupted cell membrane
Cytoplasm retained in apoptotic bodies	Cytoplasm released
No inflammation	Inflammation usually present

Table 2: ***Major differences between apoptosis and necrosis*** **(15).**

Term	Characteristic(s)
Programmed cell death	Dependent on genetically encoded signals or activities within the dying cell; a sequence of potentially modifiable events leading to the death of the cell
Apoptosis	Mediated by a subset of caspases (Fig. 1); morphology includes nuclear and cytoplasmic condensation and formation of membrane-bound cellular fragments or apoptotic bodies; not inflammatory
Autophagy	Degradation of cellular components within the dying cell in autophagic vacuoles; not inflammatory
Oncosis	Prelethal pathway leading to cell death accompanied by cellular and organelle swelling and increased membrane permeability; proinflammatory
Pyroptosis	Proinflammatory pathway resulting from caspase-1 activity leading to membrane breakdown and proinflammatory cytokine processing
Necrosis	Postmortem observation of dead cells that have come into equilibrium with their environment

Table 3: ***Terms and definitions associated with cellular death*** **(17).**

Traf2

Traf2 is a member of the Traf superfamily of proteins, all of which share certain protein structures (5). At the carboxy-terminus, the Traf proteins share a homologous region that is thought to mediate dimerization of the Traf molecules (5). *In vivo* studies of

Traf2 and other Traf proteins such as Traf6 show oligomerizaton utilizing the common Traf domain (7). The common Really Interesting New Gene (RING) domain is found at the amino-terminus of the Traf proteins and functions in an unknown manner (5). Following the RING domain, Traf proteins contain four to five zinc fingers (32). Traf proteins are found in the cytoplasm of cells and are thus thought to be adaptor proteins that associate with a variety of receptors, such as TNFα and CD40 (5). These receptors often contain death domains, such as TNFR1 associated death domain (TRADD) (39). When interacting with these death domains, Traf proteins are involved in transmitting signals downstream, often while utilizing their E3 ubiquitin ligase activity. The signaling cascades Traf proteins are involved in terminate in NF-κB activation and often serve as protection from apoptosis (39).

Traf2 has been studied extensively in mice. These studies indicate that within mice and other mammals, Traf2 exists as a single protein (54). Traf2 deficient mice are smaller and die earlier than their control counterparts and Traf3 deficient mice (54). The phenotype could be partially rescued with blocking of the TNF receptor (54). Shi *et al* looked at Traf2 in HeLa cells and found it to undergo rapid K63 polyubiquitination following TNF stimulation (43). This is a vital step allowing Traf2 to function as an E3 ligase with Ubc13 as the E2 ligase, often in pathways involved in regulating apoptosis (43).

Ubiquitination

Ubiquitination is known to play a role in controlling apoptosis and cellular protein trafficking (33). Reversible covalent modification of proteins usually takes place on Ser, Tyr, Thr, or Lys residues. Lys and Cys are the primary targets for ubiquitination. E1

ligases activate the ubiquitin molecule with ATP and a thioester link. E2 ligases carry ubiquitin to the E3 ligase for transfer to the targeted protein through another thioester linkage (Figure 6) (33). The major family of E2 proteins is known as the Ubc family, which have a catalytic core (20). The Cys residue that carries the activated ubiquitin can be found within the catalytic core. The absence of Cys constitutes the sub-family of noncanonical ubiquitin-conjugating enzyme variant (Uev). Ubc13 and Uev1 form a heterodimer. Selectivity for K63 polyubiquitin is achieved with this unique dimer of proteins (20).

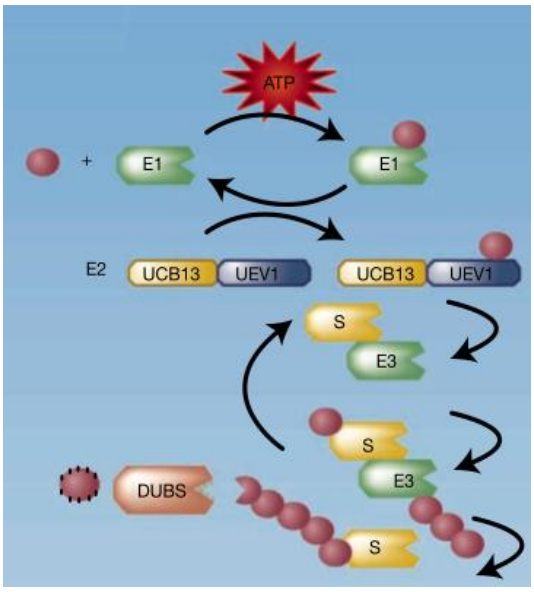

Figure 6: ***Action of E1, E2, and E3 ligases in ubiquitinating a substrate*** **(33).**

E3 ligases are a family of proteins responsible for catalyzing the addition of ubiquitin to target proteins, primarily at K48 and K63. The ubiquitin protein has multiple Lys residues, allowing polymerization of long chains (33). K48 polyubiquitination acts as a signal for proteasome degradation. The long K63 linked ubiquitin tails are thought to create a scaffold for proteins to regulate NF-κB levels. Longer chains generate a more intense signal (33).

Signaling Pathways

i. CD40 Receptor linked Activation of RelB

In the activation of RelB, a protein in the NF-κB family of transcription factors, through the CD40 receptor, the E2 ligase Ubc13 heterodimerizes with Uev1 (33, 52). The E2 activity of Ubc13/Uev1 is specific for tumor necrosis factor (TNF) receptor-associated factors 2 and 6 (Traf2 and Traf6) K63 polyubiquitination. Together, Ubc13/Uev1 and Traf2/Traf6 polyubiquitination at K63 (52). Ubc13 assembles in a first tier along with the E3 ligases Traf2 and Traf3 and cellular inhibitors of apoptosis proteins (cIAP-1 and cIAP-2) (Figure 7, box B). In unstimulated cells, cIAP-1/cIAP-2 act to polyubiquitinate K48 on NF-κB inducing kinase (NIK) in a complex similar to the first tier of assembly in stimulated cells, but not attached to the CD40 receptor (49). Activated NIK will initiate the rest of the cascade that allows for RelB activation and translocation to the nucleus. If stimulated, Traf2 acts to ubiqutinate cIAP-1/cIAP-2 at K63, a modification which changes the K48 polyubiqutination specificity of cIAP-1/cIAP-2. K63 polyubiqutitin cIAP-1/cIAP-2 catalyzes K48 poly-ubquitin tails to be added to Traf3 (49). In the second step of complex assembly, the K63 polyubiqutitinated Traf2 is thought to act as a scaffold for binding of MKK4 (a substrate for MEKK1), mitogen activated protein kinase (MAPK) protein MAP or extracellular signal-regulated kinase kinase 1 (MEKK1), and inhibitor of nuclear factor κB (IκB) kinase γ (IKKγ) (34).

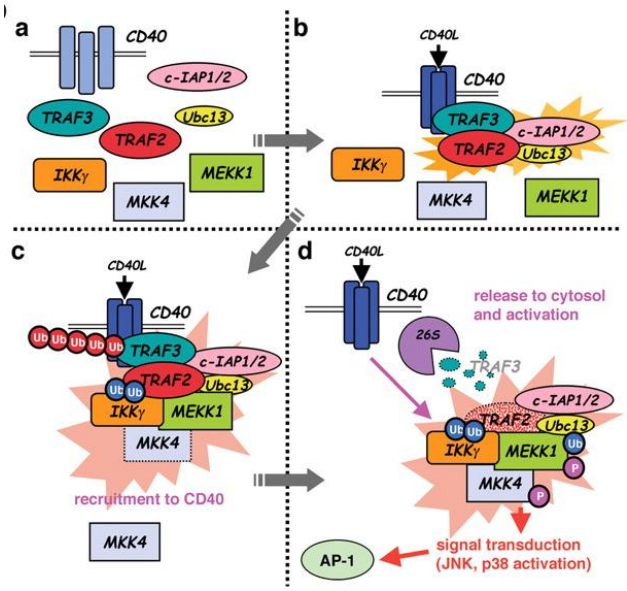

Figure 7: ***Assembly of proteins at CD40 receptor and translocation to the cytosol*** **(34).**

The completed protein complex is attached to the receptor though Traf3. Proteasome degradation of Traf3, due to K48 polyubiquitin, releases the complex into the cytosol (Figure 8). The activated complex stops ubiquitinating NIK and instead phosphorylates NIK. Phosphorylated NIK is activated and phosphorylates IKKα (49). The phosphorylation of p100 occurs at S886 and S870. Degradation and processing are signaled by K48 polyubiquitin chain catalyzed by NIK (50). The degradation of p100 generates p52. RelB dimerizes with p52 and translocates to the nucleus to activate transcription (49).

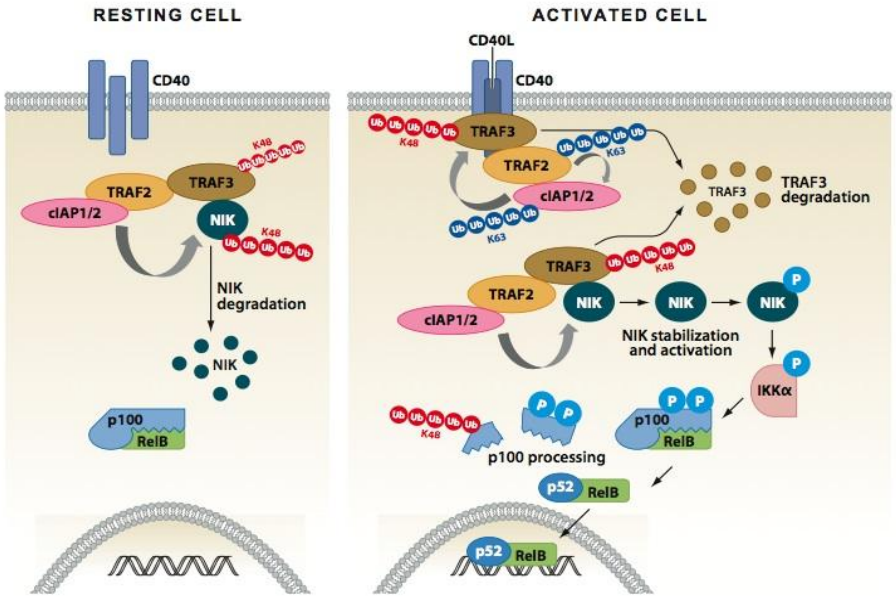

Figure 8: ***Noncanonical activation of NF-κB family member RelB thorugh the CD40 receptor*** **(49).**

ii. Toll-like Receptor 3 Activation of NF-κB

TLR3 is sequestered in the endosome and will recognize dsRNA (Figure 4) (28). Previous studies have shown it to be the only Toll-like receptor to operate independently of the MyD88-dependent pathway (28). After a virus has successfully infected a cell, the dsRNA is released and protein synthesis of viral proteins begins. Dimerized TLR3 can then bind the dsRNA imported into the endosome (3, 56). Binding of antigen to the

receptor recruits TIR domain-containing adaptor molecule 1 (TICAM1) to the cytoplasmic end of the TLR through the TIR domain on each of the proteins (38, 53, 56). Like other TLR adaptor proteins, TICAM1 (also known as TRIF) homodimerizes and is capable of signaling to activate Type 1 interferon or NF-κB (Figure 9) (28, 38). TICAM1 functions as a signaling platform, allowing a variety of proteins such as Traf3, Traf2, Traf6, NAP1 and receptor interacting protein 1 (RIP1) to bind and relay signaling (3, 56). Signaling through RIP1 activates NF-κB. RIP1 is auto-phosphorylated and polyubiquitinated by Peli1 or Traf2 at K63 and K48 (49, 56). This polyubiquitin chain serves as a scaffold for the assembly of Traf6, MAP3K, TAK1, and TAB2 (23, 56). The complex translocates to the cytoplasm and interacts with PKR which phosphorylates TAK1 (23, 56). TAK1 activates MAP kinase signaling cascades or activates IKK (56). The IKK complex phosphorylates IκB, allowing NF-κB to translocate to the cytoplasm and activate pro-inflammatory cytokines (49). RIP1 also activates caspase-8 through FADD and to promote apoptosis (56).

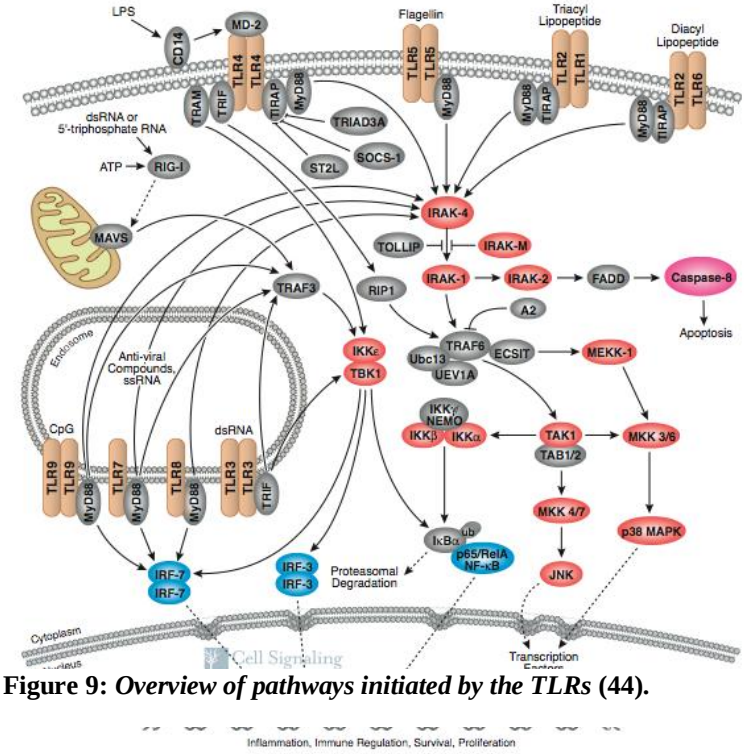

Figure 9: ***Overview of pathways initiated by the TLRs*** **(44).**

iii. Toll-like Receptor 9

TLR is localized to endosomes and recognizes viral and bacterial CpG DNA motifs found on microbial genomes (25). In unstimulated cells, TLR9 is sequestered in the endoplasmic reticulum, but after stimulation is quickly trafficked to the endosome and proteolytically cleaved (25). Proteolytic cleavage activates TLR9 and allows the receptor to bind ligand (25). Like most TLRs, TLR9 has a TIR domain to recruit the adaptor molecule myeloid differentiation factor 88 (MyD88) for signal transduction. Binding of the TIR domain of MyD88 to the TIR domain of TLR9 initiates the MyD88-dependent pathway that activates NF-κB and the MAPK signaling cascades (11, 25). The MyD88 pathway is detailed in Figure 10.

MyD88 recruits Traf6, IRAK4, and sometimes IRAK1 and IRAK2 to assemble at TLR9 (25). Traf6, an E3 ubiquitin ligase, activates the TAK1/TAB1/TAB2/TAB3 complex through K63 polyubiquitination, which in turn activates the classical MAPK signaling cascade or activates NF-κB (11). In the activation of NF-κB, the TAK1/TAB complex activates the IKK complex through phosphorylation (25). The phosphorylated IKK complex modifies the regulatory IκB molecules with a phosphate group, targeting IκB for destruction by the proteasome. Free NF-κB translocates into the nucleus and induces transcription of inflammatory cytokines (25).

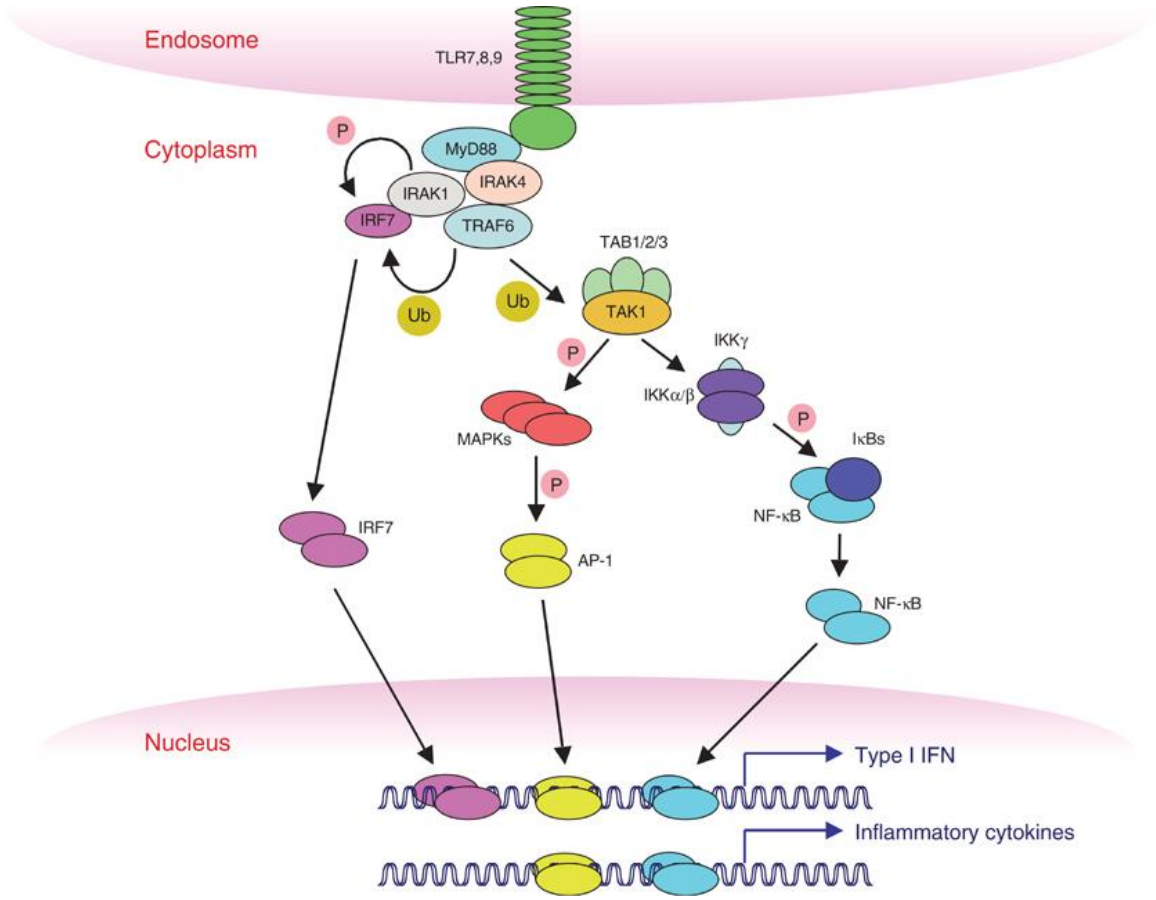

Figure 5: ***MyD88-dependent signaling cascade to activate inflammatory cytokines (26).***

iv. TNFR1 Activation of NF-κB

Tumor necrosis factor receptor type 1 (TNFR1) is activated by the pro-inflammatory cytokine tumor necrosis factor α (TNFα) (29). After stimulation, the receptor trimerizes and recruits the adaptor protein TNFR1-associated death domain protein (TRADD) to the cytoplasmic domain (49). Traf2, Traf5 and RIP1 are all recruited and

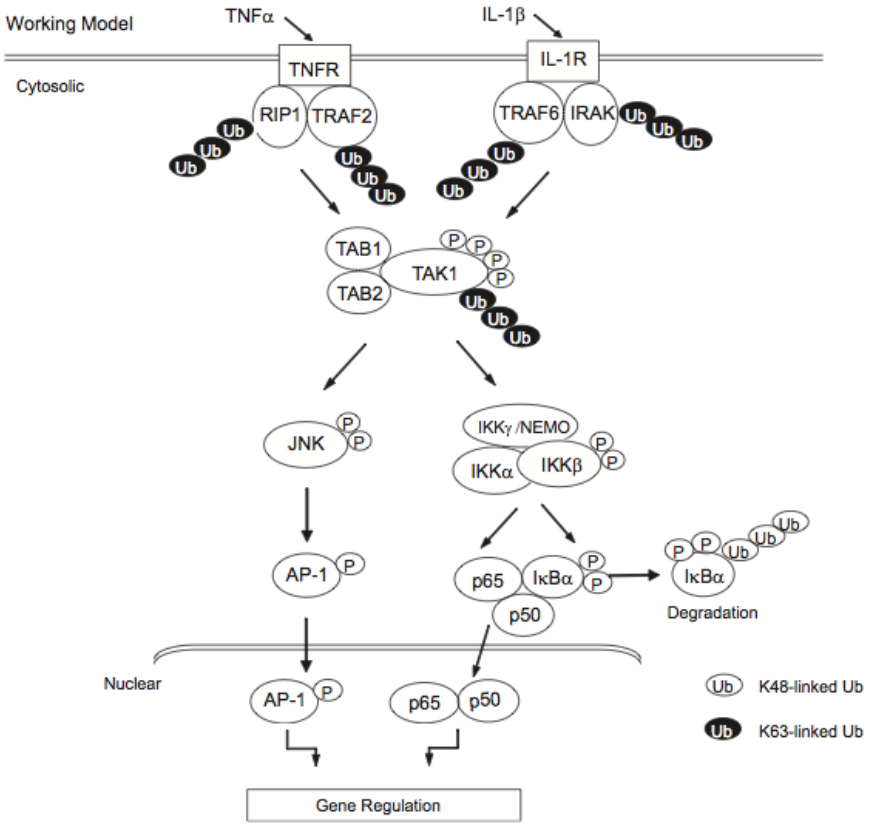

Figure 11: ***TNFR1 signaling cascade and activation of NF-κB*** **(16).**

assemble at TRADD (Figure 11) (16, 49). RIP1 is K63-linked polyubiquitinated and Traf2 is phosphorylated by protein kinase C (PKC) (30). Phosphorylated Traf2 is K63 polyubiquitinated (30). It is thought that before phosphorylated Traf2 is polyubiquitinated, it recruits IKKα and IKKβ (the catalytic subunits of the IKK complex) (30). Polyubiquitination of Traf2 promotes recruitment of a TAB/transforming growth factor-β-activated kinase 1 (TAK1) complex to the receptor complex (30). TAK1 is K63 polyubiquitinated by Traf2 (16). K63 polyubiquitinated TAK1 recruits the regulatory protein for the IKK complex, IKKγ/NEMO by providing a scaffold for binding (16). The completed IKK complex is then phosphorylated on the IKKβ subunit by TAK1 (16).

Activated IKK complex phosphorylates the IκB family members, leading to IκB degradation and release of NF-κB (49).

Genetic manipulation of the zebrafish genome

The ease of genetic manipulations in zebrafish is one of their major advantages. Multiple techniques have been developed to knockdown or knockout genes in zebrafish. Some of these techniques include: targeting induced local lesions in genome (TILLING), morpholino knockdowns, and transgenesis (35).

Mopholinos have a variety of mechanisms to block protein synthesis. The general structure of a morpholino is a twenty five morpholino base oligomer (10). These bind tightly and strongly to the RNA because of their phosphorodiamidate backbone (Figure 12, box A) (10). The two major forms of morpholinos are splice blockers (Figure 12, box B) and translational blockers (Figure 12, box E) (10). Splice blockers inhibit the formation of the splicesome complex and are usually used in zygotic embryos, whereas the translational blockers bind the untranslated region of the mRNA and are used in both maternal fish and zygotic fish.

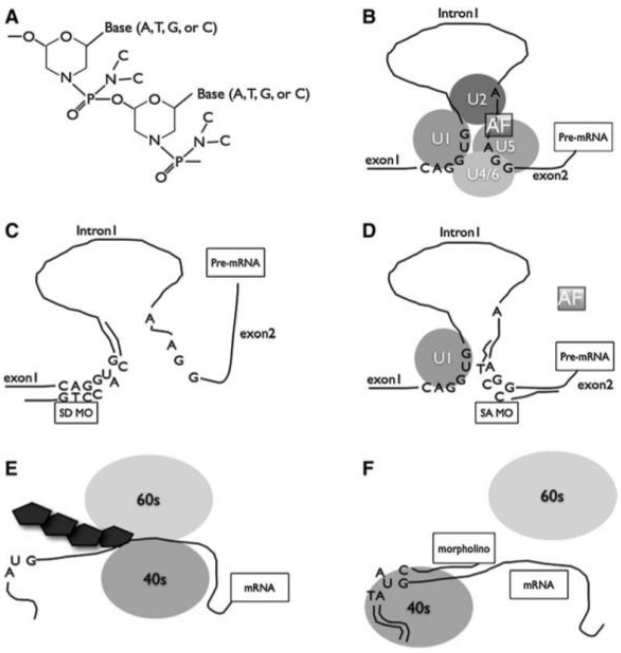

Figure 12: ***Different modes of action for morpholinos*** **(10).**

Preliminary proceedings to discover Traf2

The ubiquity of viruses in day to day life presents many challenges in health care. The immune system is the body's mechanism to not only protect itself against these invaders but to also clear infections that have taken hold. The innate immune system, composed of generalized defense mechanisms such as skin and phagocytes, works to clear infections during the first seven to ten days and is critical to mounting a strong adaptive immune response. Pattern recognition receptors known as Toll-like receptors (TLRs) are major players in recognizing the viral infection and relaying this information into the cell.

Previous data suggest that unique signaling partners may exist in zebrafish TLR pathways. Sullivan *et al*. reported several protein-protein interactions observed in the mammalian TICAM1-dependent pathway are not observed in zebrafish (47). Yet the zebrafish TICAM1-dependent pathway was capable of eliciting a similar cytokine profile for Type I IFN and NF-κB activation. Due to several known mammalian protein-protein interactions not occurring in zebrafish we hypothesized that unique protein partners must be signaling. To identify possible unique protein interactions a protein pulldown assay using MyD88 as bait was conducted. Charette *et al* (unpublished) demonstrated a unique interaction between zebrafish Traf2 and MyD88 (Figure 13).

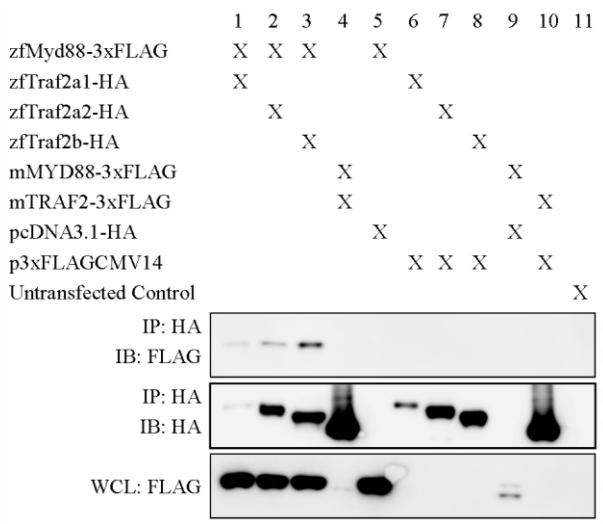

Figure 13: Each of the three paralogs of Traf2 is shown to associate with MyD88.

All three of the Traf2 paralogs

interacted with MyD88, as seen in lanes 1-3. The purpose of this study was to look at the potential roles for Traf2 in zebrafish signaling, particularly during a viral infection.

Materials and Methods

Zebrafish maintenance

Zebrafish embryos of the wild-type AB strain were maintained in petri dishes containing no more than two hundred embryos in 60 mL of egg water (60 mg/L Instant Ocean, Spectrum Brands, Madison, WI). For 6 h post fertilization, egg water was supplemented with 20 μL 0.1% methylene blue to help prevent fungal growth and bacterial infections. After immersion in methylene blue, embryos were split into plates containing two hundred embryos. Embryos were maintained in a 28 ^{o}C incubator and egg water was changed daily. Maintenance was modified from Phelan *et al* (40).

Morpholino knockdown

Morpholino injections were performed according to Bill *et al* with some modifications (10). Antisense morpholino oligonucleotides (MO) were designed by GeneTools (Philomath, OR) for each paralog of Traf2 to block splicing (Table 4). Within 1 hpf, embryos yolks were injected with 2 nL MO of each of the appropriate morpholinos using an MPPI-2 pressure injection system (Applied Scientific Instrumentation, Eugene, OR). After injection, embryos were immersed in 20 μL 0.1% methylene blue in 60 mL egg water for 5 h.

Table 4: ***Morpholinos used in this experiment*****.**

Name	Sequence	MW	Mol. Abs.	Weight	OD
TNFR1v1 constant 34	TACGTCCTTGTGCATTGCTGGCATC	8417	249090	2.53	74.73
TNFR1v2 constant 33	CTGCATTGTGACTTACTTATCGCAC	8385	254250	2.52	76.27
Traf2a1 E1/I1	CAGGACTGAATGCCGCTTACCTCAC	8389	255430	2.52	16.63
Traf2A2 E1/I1	AGATCAGCGGTGTGAATTACCCAAC	8477	264190	2.54	79.26
Traf2b E1/I1	TTTAAGAATCAAGGCTTACCACTGC	8427	263690	2.53	79.11

Immersion infections

At 3 dpf, one hundred embryos per treatment of morpholino knockdowns (the triple knockdown of all three Traf2 genes, control morpholino) added to small petri dishes. A concentration of 1 x 10^6 $TCID_{50}$ SHRV per mL^{-1} was added to the fish. After incubation for 5 h, the embryos were washed 3 times in 60 mL of clean egg water. On a daily basis for 5 days, egg water was changed and zebrafish were monitored for mortalities. This procedure was modified from Phelan *et al* (40).

Viral burden studies

Ten fish were collected and homogenized in media every 24 h. Viral burden was determined by serially diluting the filtered homogenate in EPC cells (40). After 48 h cells were examined for death. This procedure was adapted from Phelan *et al* (40).

TUNEL Assay

Ten fish were collected for each TUNEL timepoint and incubated overnight at 4 ^oC in 500 µL of 4% *para*-formaldehyde (PFA) in a 1.5 mL microcentrifuge tube. Fish

were immersed once for 5 min in 33% MeOH in PBS and 66% MeOH in PBS, and immersed twice in 100% MeOH for 5 min. The permeablized fish were then stored at −20^{o}C. This procedure was adapted from Yabu *et al* (51).

The TUNEL assay itself was adapted from Invitrogen (21). Individual fish were transferred to 1.5 mL microcentrifuge tubes and treated with 5-min washes in 66% MeOH in PBS, and 33% MeOH in PBS, followed by 2 5-min washes in (51). Fish were equilibrated in TdT buffer for 10 min (21). After 2 h-at 37^{o}C in the TdT reaction cocktail (21), the fish were washed twice for 20 min in 1x Western Blocking buffer in TBS (Roche 1921673 Brandford, CT) (12, 21). Finally, fish were treated for 30 min at room temperature in the dark with Click-iT reaction cocktail (Invitrogen) before an overnight incubation at 4 ^{o}C in 1x Western Blocking buffer in TBS (21).

Ubiquitin Assay

293T cells at 90 – 95% confluency were transfected with one of the Traf2 paralogs tagged with hemagluttin (HA), RIP1 tagged with myc, and wild type ubiquitin plasmid tagged with HA using Lipofectamine 2000 according to the manufacturer's recommendations (Addgene #17608). At 6 h post transfection (hpt), media was replaced with 100 nM sphingosine 1-phosphate. At 48 hpt, the cells were washed with phosphate buffered solution(PBS) (Invitrogen Life Technologies) and lysed in buffer containing 50 mM Tris-HCl at a pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and a protease inhibitor (Sigma-Aldrich) (48). After centrifugation at 16,000 x *g* for 15 min, the cleared cell lysate was incubated overnight at 4 ^{o}C with anti-HA agarose affinity gel resin or anti-myc agarose affinity gel resin (Sigma-Aldrich, St. Louis, MO). The whole cell lysate and immunoprecipitate were washed 3 times in buffer containing 25 mM Tris-HCl (pH 7.2),

150 mM NaCl, and 0.05% Tween 20 (48). The immunoprecipitates were eluted in 2x lane marker nonreducing sample buffer for 5 min at 100 ^{o}C (Pierce Rockford, IL) and separated on a SDS-PAGE gel (48). The separated proteins were transferred onto nitrocellulose overnight at 4 ^{o}C. Membranes were blocked with TBS (pH 7.5) with 0.1% Tween 20 and 5% nonfat dried milk before treating with mouse monoclonal primary antibody against the myc tag at 1:500 for 2 h at room temperature (Santa Cruz Biotechnology) and a secondary anti-mouse antibody linked to HRP for imaging at 1:50,000 overnight at 4 ^{o}C (Amersham Biosciences). Polyclonal rabbit anti-HA, at 1:250 was incubated for 2 h at room temperature for the other immunoprecipitate (Thermo Scientific) and the secondary goat anti-rabbit monoclonal IgG linked to horse radish peroxidase (HRP) for imaging at 1:50,000 was incubated overnight at 4 ^{o}C (Santa Cruz Biotechnology). This procedure was adapted from Sullivan *et al* (48).

Confocal microscopy

Images were analyzed with an Olympus IX-81 equipped with an Olympus FV-1000 laser scanning confocal unit using the Olympus FV-10 software package.

Results

Traf2 knockdown fish exhibit greater mortality when infected with SHRV.

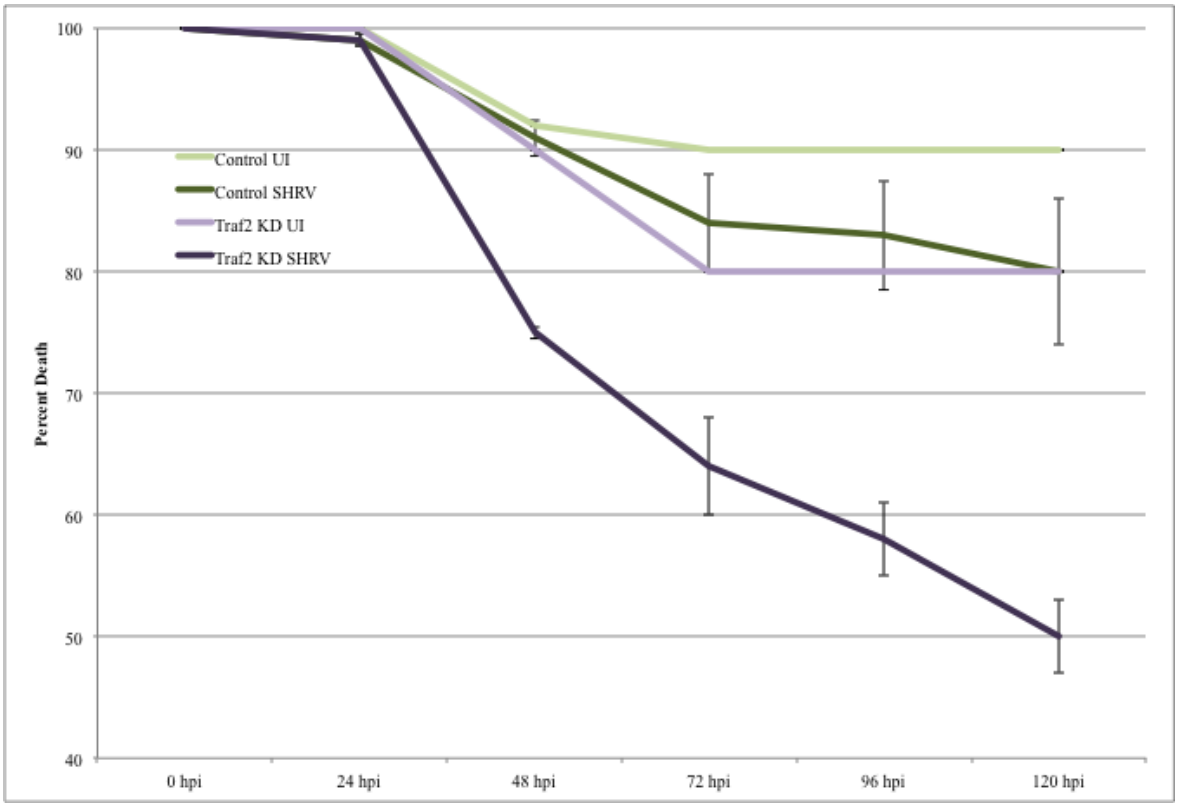

Figure 14: Triple knockdown Traf2 fish experience greater mortality than control fish when infected with SHRV. Mean mortality in Traf2 triple knockdown fish challenged with SHRV as compared to control infected fish and uninfected fish. Error bars represent the standard error between the two replicates.

Previous studies done by Yeh *et al* showed early lethality in Traf2 deficient mice and runting in the mice (54). We investigated whether zebrafish embryos showed the same runting and premature death. Zebrafish embryos were injected with the three Traf2 morpholinos at once (Table 4) at the one to two cell stages. At 3 dpf the embryos were immersed in 1 x 10^6 $TCID_{50}$/ml SHRV for 6 h. Survival was monitored over the next 5 days. Higher levels of mortalities were observed in Traf2 triple knockdown challenenged with SHRV compared to control zebrafish (Figure 14). These results indicated the during a virus infection of the zebrafish, knockdown of the three Traf2 paralogs leads to greater mortality.

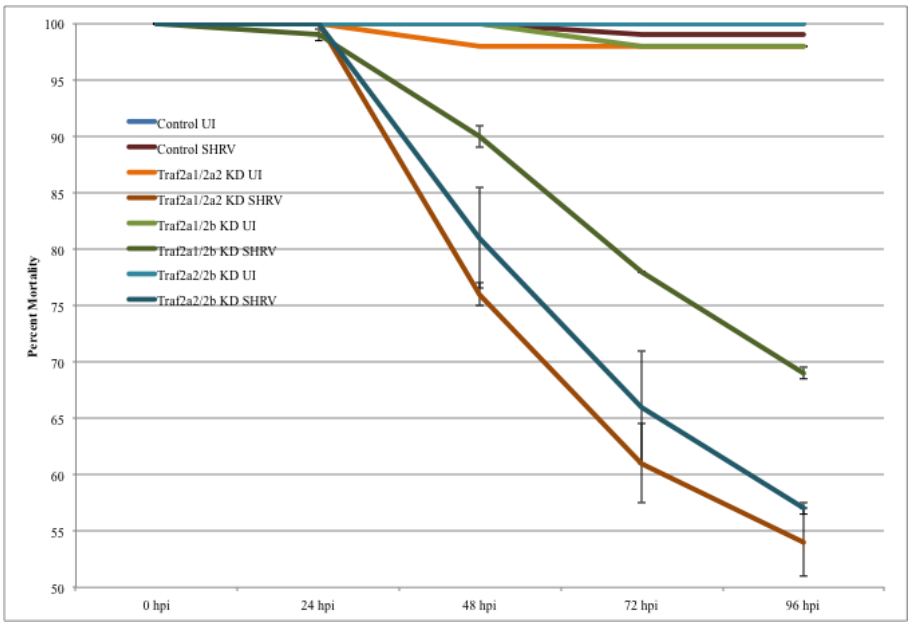

Figure 15: Pairwise knockdown Traf2 fish experience greater levels of mortality when infected with SHRV than control fish. Mean mortality levels in Traf2 pairwise knockdown fish after SHRV challenge. Error bars represent the standard error between the two replicates.

We wanted to investigate whether the increased mortality seen in Traf2 tri-morphants was due to the combined action of all three paralogs or if two served redundant and protective roles. To investigate this question, we knocked down all three pairs of Traf2 morpholinos and the individual Traf2 morpholinos (Table 3). The pairwise knockdowns of the Traf2 paralogs showed an increase in mortality over the control zebrafish as well (Figure 15). However, the single knockdowns of Traf2 paralogs did not show an increase in mortality compared to the control zebrafish (Figure 16).

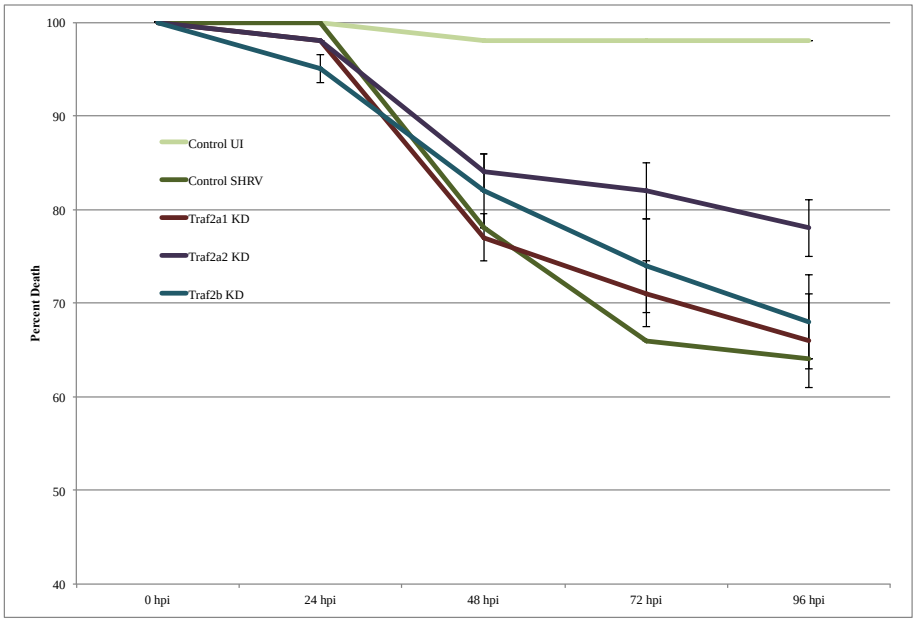

Figure 16: Knockdown of the individual Traf2 genes in fish infected with SHRV does not increase mortality as compared to control fish. Mean mortality of individual Traf2 knockdown fish challenged with SHRV as compared to control fish. Error bars represent standard error between two replicates.

The results indicate that Traf2 pairwise morphants are more susceptible to death during a viral infection, but that individual Traf2 genes are not sufficient to cause this increase in mortality during viral infection.

Traf2 zebrafish and control fish have no difference in viral burden

An increase in mortality during a viral infection is often the result of a high viral burden. To determine if this was the case in our scenario, the fish were sampled for viral

burden every 24 h for 3 days.

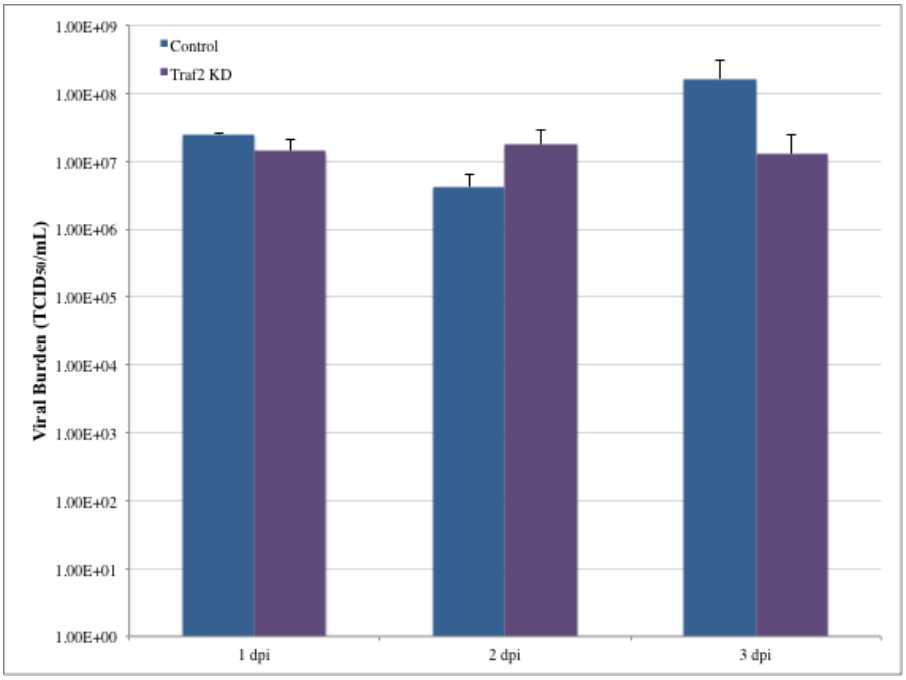

Figure 17: Traf2 tri-morphants do not have a greater viral burden than control fish. Mean viral burden in SHRV challenged tri-morphant Traf2 zebrafish.

There was no significant difference in viral burden between the triple Traf2 knockdown fish and the control fish (Figure 17). This indicates that although Traf2 morphants exhibit a higher mortality during viral infection, the increase in mortality is not due to an increaed amount of virus infecting the fish.

TNFR1 knockdown does not improve mortality seen with Traf2 knockdowns

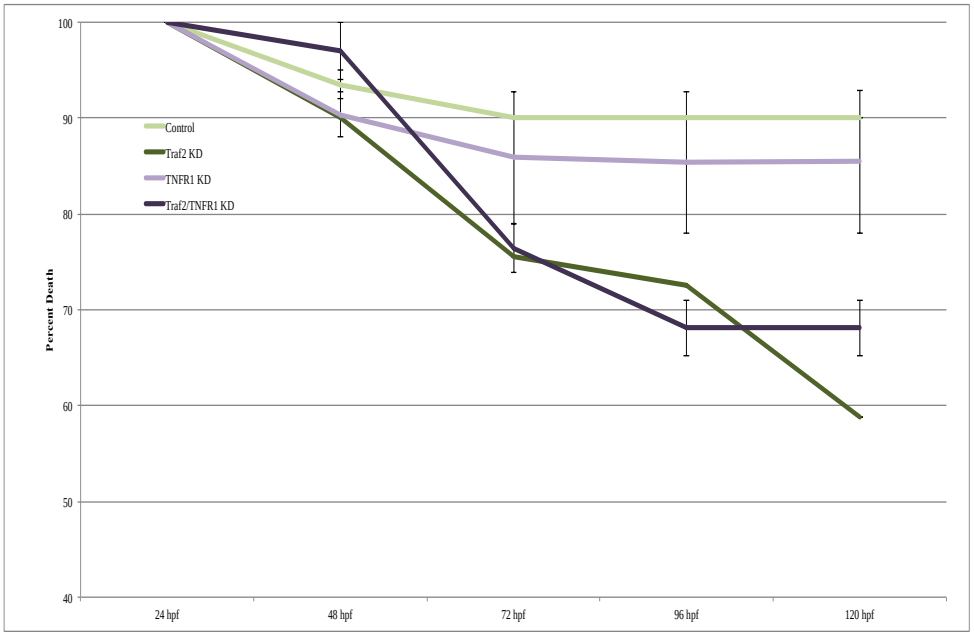

Figure 18: Eliminating the receptor TNFR1 does not increase survival in Traf2 triple knockdown fish. Mean mortality of Traf2/TNFR1 morphants as compared to controls. Error bars represent standard error between two replicates.

To determine the possible pathways that Traf2 is involved in, the TNFR1 receptor was knocked down alone or with the three Traf2 paralogs. It has been shown that Traf2 has a protective role in the TNFR1 pathway (37). By removing the receptor, there was an increase in survival of the mice in the study done by Nguyen *et al* (37). Few fish survived until the third day for infection with SHRV, so the fish mortality while uninfected was monitored from 24 hpf on until 120 hpf. We did not, however, see an increase in survival when we knockdown Traf2 with TNFR1 (Figure 18). Bates *et al* were able to use the TNFR1 morpholinos with success in their own experiments, though they were not using the morpholinos in conjugation with the Traf2 morpholinos (6). Thus the lack of rescue in the zebrafish when using the TNFR1/Traf2 tri-morphants may be because the zebrafish Traf2 proteins are not as intimately involved in the TNFR1 signaling pathway as mouse

Traf2 is in the mouse TNFR1 pathway. The results from this experiment do not show an increase in survival in Traf2 tri-morphants that are also deficient in TNFR1.

Traf2 may increase apoptosis, which may lead to increased mortality

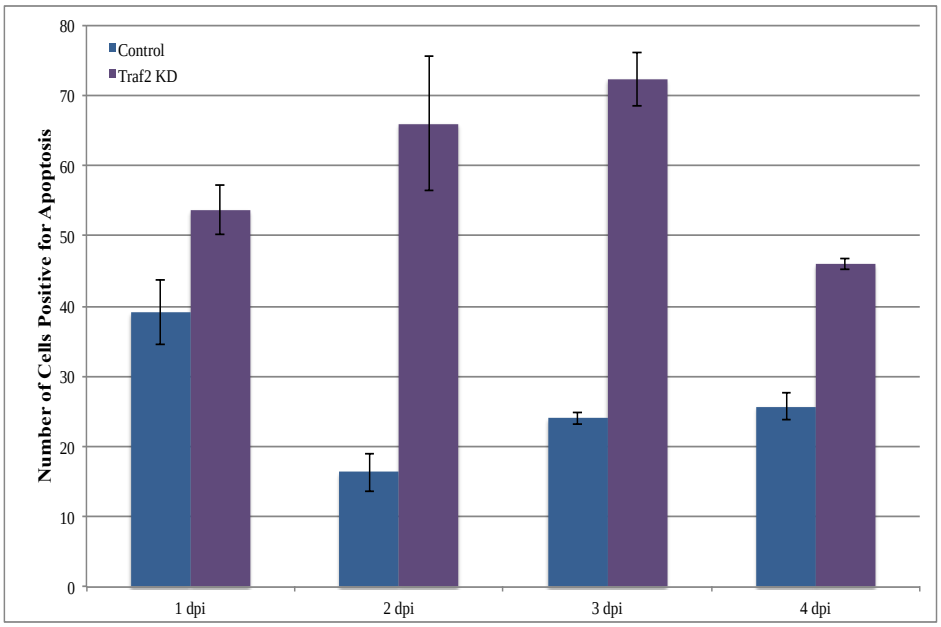

Figure 19: Triple knockdown Traf2 fish infected with SHRV experience greater levels of apoptosis than control infected fish. Mean number of cells undergoing apoptosis in Traf2 tri-morphants challenged with SHRV. Error bars represent standard error between the three fish sampled.

Previous studies have indicated that Traf2 negatively regulates apoptosis through the Caspase-8 pathway (19). To look at the possible role of Traf2 in preventing apoptosis in the zebrafish, we analyzed SHRV infected fish at 1 – 4 dpi via the TUNEL assay. The TUNEL assay attaches a fluorescent probe to the nicked ends of DNA. A characteristic feature of cells undergoing apoptosis is DNA fragmentation, a process that leaves lots of ends of DNA open for TUNEL staining (36). TUNEL stained fish were analyzed by confocal microscopy. Cells undergoing apoptosis were counted in each fish, though results were inconsistent. In general there were more apoptosic cells in the Traf2 knockdown fish than in the controls, however the difference in number of apoptotic cells was not statistically significant (Figure 19).

Traf2 as E3 ubiquitin ligase

In mammalian systems, Traf2 acts as an E3 ubiquitin ligase, ubiquitinating cIAP-1/cIAP-2 at K63 (49). To investigate whether the three Traf2 paralogs in zebrafish could function as an E3 ubiquitin ligase, 293T cells were transfected with plasmids containing the Traf2 paralogs, RIP1, and ubiquitin, (Figures 20 – 22) (31). The cells were lysed and immunoprecipitated for the myc tag. This targests RIP1, a potential target for E3 ubiquitination by Traf2. A second experiment immunoprecipitated for the HA tag, targeting the ubiquitin molecules and the Traf2 paralogs. Traf2a2 is expressed by the transfected 293T cells, but the other two Traf2 paralogs are not. This may be due to poor expression of Traf2a1 and 2b and is a problem we are actively still investigating. Traf2a1 and 2b could also be masked by high expression of ubiquitin. RIP1 expression was also observed in expressed transfected 293T cells (Figure 21). RIP1 did not produce smearing that is characteristic of polyubiquitinated proteins (Figure 20), leading us to believe that polyubiquitination is not occurring between the individual Traf2 paralogs and RIP1. RIP1 is not being ubiquitinated in these cells as the immunoprecipitate for HA blotted for the myc tag yielded no product.

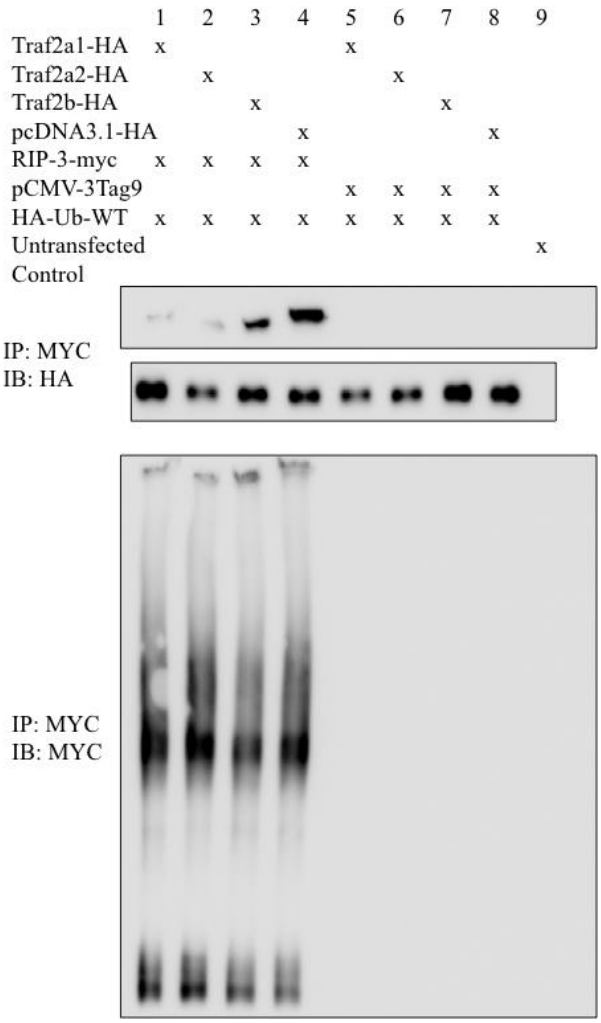

Figure 20: RIP1 is not ubiquitinated by Traf2. Immunoblot of cell lysate immunoprecipitated for the myc tag present on RIP1. The top box was blotted for the HA tag present on the Traf2 proteins and ubiquitin, while the bottom box was blotted for the myc tag.

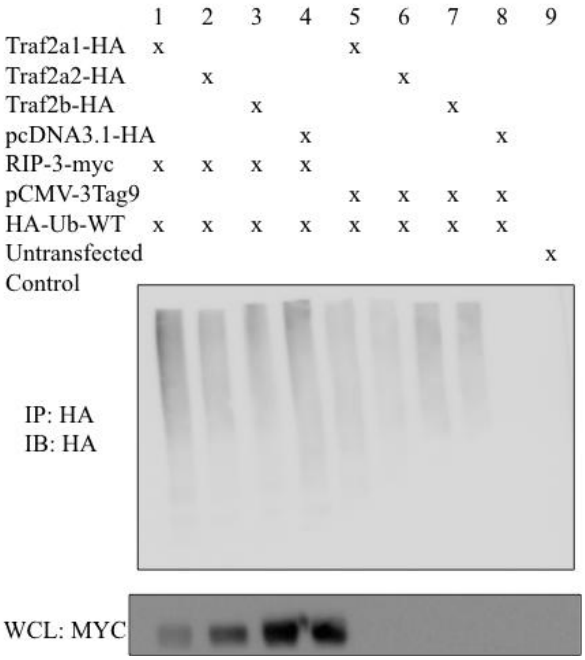

Figure 21: Ubiquitin is most likely present in the cells. The top box is an immunoblot of cell lysate immunoprecipitated for the HA tag present on ubiquitin and the Traf2 proteins and blotted for the HA tag present on the Traf2 proteins and ubiquitin. The bottom box was blotted the whole cell lysate blotted for the myc tag.

	1	2	3	4	5	6	7	8	9
Traf2a1-HA	x				x				
Traf2a2-HA		x				x			
Traf2b-HA			x				x		
pcDNA3.1-HA				x				x	
RIP-3-myc	x	x	x	x					
pCMV-3Tag9					x	x	x	x	
HA-Ub-WT	x	x	x	x	x	x	x	x	
Untransfected Control									x

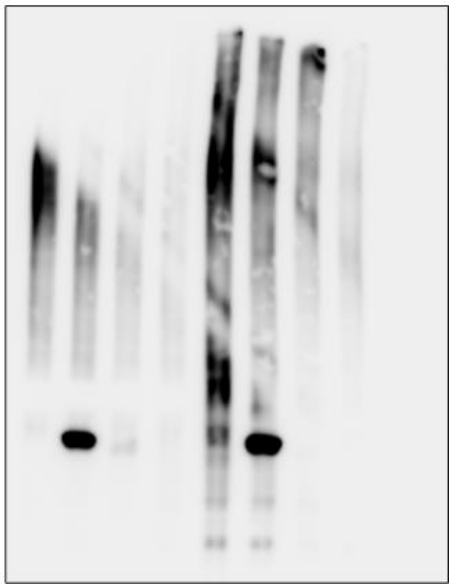

Figure 22: Traf2a2 is the only Traf2 paralog to be expressed in the blot. Immunoblot of the whole cell lysate for the HA tag present on ubiquitin and the Traf2 proteins.

Discussion

Traf2 is critical in regulating apoptosis in mammalian systems. It acts as an E3 ligase to poly-ubiquitinate cIAP-1/cIAP-2 at K63 (34, 49). In mice, Traf2 deficiency caused runting and early death due either to TNF receptor lethality or increased apoptosis (54). In mammalian systems, Traf2 exists as a single protein involved in a variety of pathways, including TLR3, TNFR1, and many others with death domains (39). Zebrafish are unique and possess three Traf2 paralogs: Traf2a1, Traf2a2, and Traf2b. The potential for unique interactions with other proteins like MyD88 (Figure 13) makes research with the zebrafish Traf2 proteins vital to understanding the alternative pathways and mechanisms organisms use to protect against inflammation and apoptosis.

Previous studies using mice as a model demonstrated that Traf2 deficiency leads to early mortality without infection (54). If zebrafish experience lethality in the same manner as mammalian systems, it will provide insight into the pathways that utilize the zebrafish Traf2 paralogs. The Traf2 tri-morphants infected with SHRV experiences greater levels of mortality than the control fish (Figure 14). This may be due to Traf2 involvement in anti-apoptotic pathways, perhaps acting as

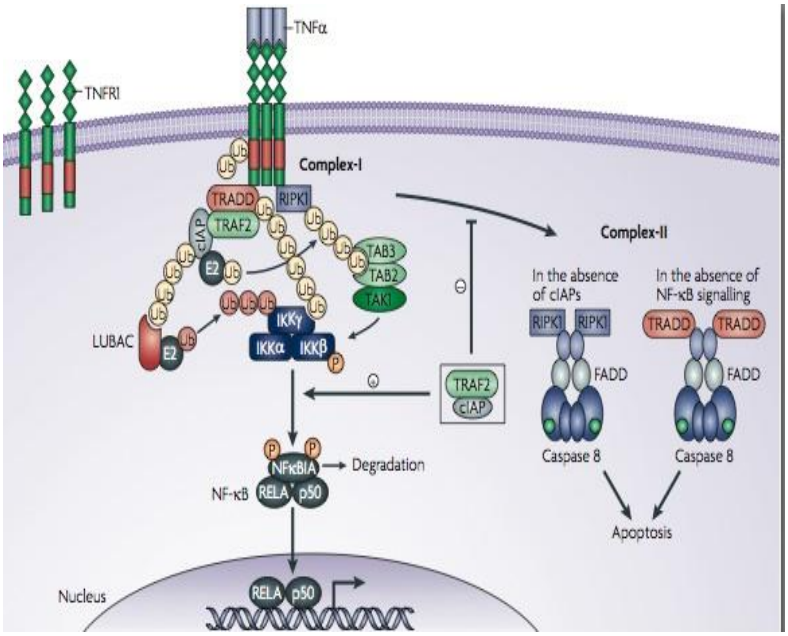

Figure 23: TNFα signaling pathway to inflammatory cytokines or apoptosis (19).

mlevels of mortality in fish prior to infection that were between 24 and 72 hpf were observed (Figure 18). In Figure 24 we can see that Traf2 works to inhibit apoptosis through the Caspase-8 pathway. By removing Traf2, there is potential for greater apoptosis without that level of regulation. Apoptosis will occur because Traf2, which is a negative regulator of the Caspase-8-dependent pathway, has been removed, allowing the pathway to proceed. Greater levels of apoptosis could cause an increase in mortality. Mice deficient in Traf2 showed early lethality and runting, similar to the effects seen in the zebrafish (54). Yeh *et al* determined that this early mortality was not just due to Traf2 playing a role in TNFR signaling, but that Traf2 must also be vital in other signaling pathways such as TLR3 signaling and signaling through the CD40 receptor (54). These findings are consistent with our findings in the zebrafish. The pairwise knockdown fish experience similar levels of mortality rates to each other, from 50 – 70 % survival (Figure 15), suggesting that at least two of the paralogs of Traf2 are required for function. When paired with the results of the single Traf2 morphants where no increase in mortality is observed (Figure 16), this suggests that zebrafish Traf2 may be forming heterodimers or even heterotrimers, a result consistent with previous studies have shown demonstrating that mammalian Traf2 homodimerize (43).

The increase in mortality observed in virus infected Traf2 morphants may be due to the inability to clear the virus. The viral burden was assessed in both Traf2 tri-morphants and control fish and there was no significant difference in viral burden (Figure 17). Therefore, the increase in mortality is not due to an increase in viral burden. Traf2 is not a key player in a pathway that clears virus.

Since viral burden is not the cause of the increase in mortality, we looked at the levels of apoptosis in the fish. In mammals, Traf2 is a negative regulator of apoptosis in Caspase-8 pathway (Figure 23). Utilizing the TUNEL assay to label apoptotic cells *in vivo*, SHRV infected Traf2 tri-morphants and control fish were compared over 4 days. A moderate increase in apoptotic cells in the Traf2 tri-morphants was observed (Figure 18), though these numbers varied widely between replicates. The greatest increase in apoptosis occurs between two and three days post infection. The apoptosis seen in the fish was localized mostly along the fin of the tail and could be due to developmental apoptosis or normal cell death on the skin edge. The Traf2 tri-morphants did have some apoptosis in the eye region; this was not significantly different than the control fish. The slight increase in apoptosis in the Traf2 tri-morphants could also be caused by off-target effects of morpholinos, as not all morpholinos have the same effects on the developing embryos (10). It is entirely possible that the Traf2 morpholinos have the potential to cause more p53-induced apoptosis than the control morpholinos, particularly since we saw apoptosis in the eye region, which is characteristic of the off target effects (10). Reimers *et al* used the TUNEL assay in conjugation with an acridine orange stain to depict apoptosis in ethanol stressed zebrafish embryos (41). Acridine orange stains cells undergoing death in general, and does not allow differentiation between necrosis and apoptosis. For this reason, we chose to only use the TUNEL assay, which is specific for apoptosis through the tagging of nicked DNA. Reimers *et al* were able to show apoptosis in embryos of a similar age range, in much greater quantities than we were able to achieve (41). Reimers' group may have been more successful in their numbers because they were not co-injecting with the Traf2 morpholinos or because they did not need as

great of numbers of zebrafish embryos to survive. For infection/mortality studies, we need a greater number of fish to survive until the third day post fertilization than for studies focusing just on mortality. Because results were inconsistent, we cannot definitively say that the increase in mortality is caused by the Traf2 knockdown, but it may be a contributing factor.

Because simultaneous removal of the TNFR1 receptor with Traf2 rescues the effects in Traf2 deficient mice, the TNFR1 receptor was knocked down in the zebrafish (54). However, there was not a significant increase in survival of the Traf2 tri-morphants. In zebrafish, unlike mice, there are two TNFR1 receptor genes: TNFR1v1 and TNFR1v2. The TNFR1 morpholinos were originally described by Bates *et al* in zebrafish (6). The group showed TNFR1 deficient fish were more resistant to lipopolysaccharide exposure, a potent toxin (6). Simultaneous knockdown of TNFR1v1, TNFR1v2, Traf2a1, Traf2a2, and Traf2b resulted in no change in mortality as compared to the Traf2 tri-morphants (Figure 18). Knockdown of TNFR1v1 and TNFR1v2 may have rescued the Traf2 tri-morphant high mortality phenotype but the high amount of morpholino that was injected per embryo may have caused off-target effects. Morpholinos have the potential to cause side effects such as enlarged eyes, swollen yolk sacks and early death (10). The lack of rescue in our studies may be because of a difference in the TNFR1 pathways of mice and zebrafish. Though Traf2 is involved in the mouse TNFR1 pathway, the zebrafish may use an alternative method of signaling. Incomplete rescue of the Traf2 tri-morphants may also be due to cross-talk amongst different pathways. For instance, in mammalian systems both TLR3 and TNFR1 utilize RIP1 in downstream cascades (13). RIP1 is polyubiquitinated and eventually leads to NF-κB activation in both the TLR3 pathway

stimulated by dsRNA and the TNFR1 pathways stimulated by TNFα (13). Using just TNFR1v1 knockdown simultaneously with the Traf2 tri-morphants did not rescue the mortality. There were still off-target morpholino effects, which could be because of the effects of the other TNFR1 gene still present.

In mammalian systems, Traf2 has been shown to function as an E3 ligase that K63 poly-ubiquitinates cIAP-1/cIAP-2 and other proteins (34, 49). We looked at the potential for each of the three Traf2 paralogs to play a similar role with RIP1. Previous studies have shown that mammalian Traf2 function as an E3 ligase with Ubc13-Uev1a as the E2 ligase to polyubiquitinate RIP1 at K63 on RIP1 (4). When RIP1 is polyubiquitinated the tail of ubiquitin acts as a scaffold for other signaling molecules and complex formation (16). Alvarez *et al* found that sphingosine-1-phosphate is necessary for this reaction to take place (4). In our own transfection, we did used sphingosine-1-phosphate and RIP1 as our potential target for ubiquitination. We did not however, see ubiquitination of RIP1 (Figures 20, 21). Traf2a2 and RIP1 were strongly expressed in the cells (Figures 20, 22). The other proteins may not have been expressed in the cells. To determine if Traf2a1 and Traf2b are viable for expression in the cells, sequencing would need to be done. In Figure 22 we do see the characteristic smearing of ubiquitin, but because both ubiquitin and Traf2 have the same HA tag we cannot definitively say Traf2a1, Traf2a2, and Traf2b are being masked by ubiquitination of something else in the cell. The three Traf2 paralogs were transfected separately and this could be part of the reason ubiquitination did not occur. Previous studies show that Traf2 oligomerizes with itself and other members of the Traf superfamily utilizing the Traf domain (7). Perhaps the existence of three Traf2 proteins in zebrafish means that for fully functional ability at

least two of the paralogs must be present to complex together, or for all three even to be necessary. Based on our own mortality studies earlier, where we saw a decrease in survival when two or more Traf2 paralogs were knocked down as opposed to the single Traf2 paralog morphants whose survival was not significantly different than control fish, it could be surmised that just two are necessary. With what we have seen so far, it is inconclusive whether the Traf2 paralogs are acting as E3 ubiquitin ligases for RIP1.

Further experiments are necessary to determine if the zebrafish Traf2 paralogs act as ubiquitin ligases. In particular, I would try a transfection with all three paralogs in one set of cells with RIP1. Other transfections with potential targets for ubiquitination such as TAK1 would show if Traf2 in zebrafish is just targeting something else. The conditions of the transfection could also be modified. For instance, it would be beneficial to change the HA tag on ubiquitin to something else. With the current tag, our immunoprecipitate for HA may have become too saturated with ubiquitinated molecules to hold any of the Traf2 proteins. It would also be interesting to try another rescue of the phenotype seen in the Traf2 tri-morphants. This might be achieved by knocking down a different potential receptor, such as TLR 3 or TLR 9 and infecting the fish. Part of our difficulties in the experiments has been getting the fish to survive long enough to have a sufficient number for experiments at an older age. Co-injecting with the p53 morpholino as suggested by Bill *et al* could help to eliminate some of these off target effects (10).

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